

**CONTINUOUS FLOW, HIGH-THROUGHPUT MICROFLUIDIC PLATFORMS
FOR GENOMIC DISCRIMINATION ASSAYS**

by

Lingshu Liu

A thesis submitted to Johns Hopkins University in conformity with the
requirements for the degree of Master's of Science and Engineering

Baltimore, Maryland

June, 2015

ABSTRACT

DNA technologies from PCR to allele discrimination are now common and indispensable techniques utilized in a myriad of fields such as healthcare and agriculture. While traditional bench top methodologies are well refined, the standard techniques often require sample volumes and costs that are prohibitive for high throughput applications. Thus genetic screening at rapid rates and low costs is a requirement for further propagation of DNA technologies in large scale operations. Microfluidic platforms are particular well-suited to meet the challenge of creating technologies capable of high throughput, continuous flow, multiplexed allelic discrimination. Specifically, by reducing a typical reaction system from a milliliter scale Eppendorf tube down to nanoliter sized droplets, genetic screening may be performed at a fraction the cost. Here, we present two novel poly(dimethylsiloxane) (PDMS) microfluidic platforms capable of multiplexing single nucleotide polymorphism (SNP) detection. Utilizing unique SNP assays (Invader and Taqman PCR) well suited for microfluidics applications, both platforms include on-chip optical detection of fluorophores that allow for direct allelic read out. Utilizing benchtop amplified target DNA, successful SNP detection on-chip was achieved in the first device with unambiguous signal readout spanning nearly 80 target DNA/probe combinations. In the second device, both target DNA amplification and allele detection were performed on-chip. Taken together, our novel PDMS microfluidic platforms provide a key advance in microfluidic devices for allele discrimination. Device capable of high throughput and affordable genomic screening now looms.

Thesis readers: Tza-Huei (Jeff) Wang, Ph.D.; Kevin J. Yarema, Ph.D.; Zachary Gagnon, Ph.D.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my Master's project and thesis advisor, Dr. Tza-Huei (Jeff) Wang, for his expertise and guidance. It was only with his constant encouragement that these projects were able to flourish. I will forever be grateful for Dr. Wang's decision to take me in as one of his own students.

Asides from my Master's advisor, I would also like to thank my undergraduate research advisor, Dr. Kevin Yarema, for all of his support. From class to lab, Dr. Yarema never failed to amuse me with new and interesting facts from glycobiology to the origins of Groundhog Day. I am extremely grateful for my time and all that I have learnt from my time in Dr. Yarema's lab.

Within the lab, I would like to thank my wise and humble mentor, Dr. Wen Hsieh. Without Wen, these projects would have no doubt taken weeks, if not months, longer to progress to where they are today. His endless patience and demand for high standards of work have taught me to do the same, and in return have made me a better researcher. I will miss the lengthy late night philosophical discussions with Wen about science and life. I would also like to thank graduate students Helena Zec and Anu Kaushik. Helena, for not just her wealth of knowledge about the Invader project and device fabrication, but also her ability to always bring laughter and fun to even the most tedious of experiments. I may even miss running those sixteen hour multiplexed Invader reactions on chip. Anu, for his hard work towards building the heating platform and letting me claim the optical detection set up for days on end to meet deadlines. Lastly, I would like to thank my fellow lab mates Andy, Alex, Dr. Liu, Sarah, Liben, Dong Jin, Ye, Chrissy, Natt and Dr. Tom Pisanic for making the lab such a great environment to work in.

As the sponsor of our project, Pioneer DuPont has offered an abundance of cooperation and expertise from the assay development standpoint. I would like to thank them for their input and advice.

Lastly, I would like to thank my family and friends for the unconditional love and support they have given me. Specifically, I would like to thank Billy Kang, for the encouragement, support and late nights spent reviewing concepts and tackling assignments with me.

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CHAPTER 1:

Introduction

1.1 The Rise of Microfluidics

The smaller, the better. In today's world, the transition from macro down to micro scaled technologies is a prevalent driving force behind many disciplines from biochemistry to computer engineering. The initial drive for miniaturization in the 1980s gave birth to the field of microelectro-mechanical systems (MEMs) which used silicon-based devices to study thermal, electrical and mechanical behaviors in the sub-micron scale [1]. At around the same time, traditional chemical analysis techniques requiring expensive and bulky equipment such as chromatography and electrophoresis were first achieved in miniaturized systems composed of capillaries and microcanals [1]. It was this wedding between microfabrication and chemical analysis that gave birth to the concept of miniaturization of bioassays on microfluidic platforms. In particular, key advances in the field of optics had allowed for detection systems in molecular analysis to achieve much higher resolutions and sensitivities [1]. The explosive growth in the field of molecular biology and genomics in the 1980s necessitated the need for technologies that were capable of performing high-throughput reactions at affordable costs. As a result of this, microfluidics – the field of micro to femtoliter sized fluidic systems – rose to meet these challenges. As we progress further into the age of information, the miniaturization of electrical components have allowed for faster data acquisition and processing. Similarly, as we mature in our understanding of molecular biology behind genomic assays and assay engineering, the

miniaturization of fluidic systems and bench top technologies have allowed for microfluidic devices capable of high throughput genomic data analyses.

1.2 The Development of Fluorescence-based Genetic Screening Microfluidic Devices

Closely following the expansion in the field of molecular biology, microfluidic platforms for the purpose of genetic screening have allowed for the downscale of classic assays from Eppendorf tubes and 96 well plates down to nanoliter droplets. In essence, traditional bench top assays in laboratories were shrunk to palm-sized microfluidic chips. By reducing the volumes and scales of the milliliter reactions by a million fold to nanoliters, microfluidics offers several distinct advantages over traditional methods. Studies that were once limited by the need for high-throughput screening and precise optical measurements could now be approached from the microfluidics perspective to perform the same screening functions at many times the speed and a fraction the cost. Specifically, droplet microfluidics confines samples and even entire reaction to droplets isolated by phase separations. In this way, a carrier oil phase separating aqueous droplets through hydrophobic-hydrophilic interactions could be used to generate endless combinations of independent reaction systems in a rapid and inexpensive manner [2].

Droplet microfluidics has tremendous potential for the amplification and detection of nucleic acids and genetic markers. Genetic studies start from the cellular level with isolation followed by DNA extraction. The ideal chip would be capable of performing sample isolation, extraction, purification, amplification and detection in a streamlined, continuous flow manner. As such, platforms designed for nucleic acid amplification must meet several considerations for the required mechanical and biochemical reactions.

However, due to many challenges that arise in each sample processing stage alone, effort has been placed into developing microfluidic devices capable of performing one or two of the aforementioned capabilities.

Due to its origins from MEMs, microfluidic devices were largely fabricated from silicon in the earliest studies. Silicon's cheap price and semiconductor properties had made it a popular choice for MEMs. Thus, fabrication techniques involving silicon have been very well characterized. The earliest studies that sought to use microfluidics for genetic amplification and quantification were carried out on silicon. Silicon channels were produced using chemical and physical etchants and often the devices relied on downstream, off chip electrophoresis for read out and genotyping [3, 4]. However, silicon as a base material presented one major drawback for on-chip read out. Silicon's strong absorption spectrum within the UV/vis spectrum makes it a poor choice for microfluidics platforms designed to detect fluorophores with emission spectrums within the UV/vis region. In lieu of silicon, poly(dimethylsiloxane) (PDMS) has become a popular base material. PDMS has several advantages over silicon. Its transparency in the UV/vis region is compatible for optical detection with fluorophores in that range. Furthermore, it is a soft material, and therefore is amendable to shaping and also bonding with other surfaces [5]. PDMS based microfluidic systems was made possible with the development of soft lithography techniques, and this will be the main method of fabrication employed in all devices described in this thesis [6, 7].

Fluorescence based detection on microfluidic devices began originally as a means of visualizing, tracking and sorting microfluidic droplets. It was not until the early 2000's that fluorescent molecules were coupled to DNA molecules for on-chip sequencing [8].

The approach of the late 2000's marked the first instances of microfluidic devices capable of on-chip thermocycling in microfluidic droplets on silicon [9]. This system had used flow focusing to generate droplets, then the chip containing the static droplets was thermocycled on a Peltier heating system. A Nikon camera equipped with fluorescent filters was used to capture the time evolution of the fluorescence signal over each cycle. The ability for on-chip thermocycling then led to on-chip, continuous flow droplet PCR to be demonstrated in PDMS [10]. In this design, the microfluidic chips were fabricated through the soft lithography methods pioneered by Whitesides et al. [7]. Droplets formed from flow-focusing were sent through a series of incubation channels on chip that experienced different temperatures. The lengths of the incubation channels at each thermal zone corresponded to specific annealing, denaturation and extension times in the PCR cycle. On-chip read out was achieved by narrowing the incubation channels and using laser excitation to detect single droplet fluorescence signals. Using this continuous flow idea, many other studies have spurred the growth of microfluidic droplet PCR devices for genetic amplification and screening. All devices designed and fabricated in this thesis are based off of soft-lithography using PDMS.

1.3 Microfluidic Technologies for Agriculture

An emerging concern in recent decades that has become evident as the world's population reaches an unprecedented high. Technological advances and specifically, advances in medical care and population health, have continually increased the life expectancy from 47 years to 73 years in recent years [11]. The prolonged life expectancy together with higher birth rate over the last decade steadily increased the population size.

Of the numerous studies performed on population growth predictions, the one consistent conclusion is that the world's population will continue to grow until subsequently, finite resources such as land and most importantly food will become the limiting factor [12, 13]. Thus appraised, there is a pressing need to develop technologies that are able to aid the agriculture industry in keeping up with this growth demand. Advances in genetic breeding and marker assisted selection (MAS) have made the plant breeding process more efficient with higher yields [14]. The consequence of this however, is the high cost and limited speed in screening hundreds of thousands of crops to find the desirable genetic trait (allele); and in this light, microfluidics proves to be an ideal solution to this problem. Specifically, microfluidic technologies offer the ability to simultaneously sample an array of marker probes against multiple samples (multiplexing), perform the reaction, and provide end point read out through continuous flow at sampling rates of up to 1 kHz [15].

This thesis describes the fabrication and testing of devices for the general aim of creating a novel, microfluidic droplet based platform capable of performing multiplexed, high-throughput screening of alleles. Specifically, the assays translated onto chip all function to identify signal nucleotide polymorphisms (SNPs), and the eventual goal is to achieve a means of screening for SNP governed alleles directly from an existing genomic sample without need of pre-amplification on bench top.

CHAPTER 2:

Allele Discrimination Using On-chip Invader Assay

In this chapter, we describe in detail a robust microfluidic device for conducting continuous flow, on-chip multiplexed Invader assay on synthetic and genomic DNA extracted from maize. First, the microfluidic chip was fabricated from PDMS through soft lithography to operate using pressurized valves atop a heating system. Next, bench top verification of the Invader assay was performed with all synthetic and genomic samples, and those products loaded on the device to test for optical detection. Lastly, the Invader assay was both performed and detected on chip with synthetic targets and genomic DNA targets through on-chip multiplexing of eight DNA samples with ten probes. End point measurements were made using confocal fluorescence spectroscopy and an allelic call outs were made that corresponded well with results obtained off chip.

2.1 The Invader Assay and Single Nucleotide Polymorphism Detection

The Invader assay is an isothermal reaction that uses a flap endonuclease (FEN) to cleave specific structures formed by a target SNP in a sequence. The assay consists of two reaction steps: an initial SNP recognition step and a subsequent fluorescent probe removal step. The first step of the Invader assay revolves around three major components: FEN, the Invader probe and the signal probe containing the flap. During isothermal incubation, a tripartite structure (**figure 2.1.1**) forms between the target DNA and the two oligonucleotide probes. If the target DNA contains the correct SNP that corresponds to the

signal probe, the correct tripartite structure formed will be recognized and cleaved by the FEN. Upon FEN cleavage, the flap region on the Invader probe will be free to move onto the optical reporting step of the assay. The liberated flap region combines with a fluorescence resonance energy transfer (FRET) cassette and forms a similar tripartite structure that is recognized by FEN. The FRET cassette consists of two fluorophores (a donor and a quencher) forming a FRET pair. Under normal conditions, a weak donor signal is observed due to quenching from the proximity with the fluorophore. Upon recruitment of the liberated Invader flap and FEN to the FRET cassette, the donor fluorophore is cleaved and freed from the quencher. In this manner, the FRET cassette releases freed fluorophores and the fluorescence signal indicates the presence of the corresponding SNP in the DNA target. In the case of a DNA target that does not contain the SNP in question, the tripartite structure does not form correctly and FEN is unable to undergo secondary cleavage of the FRET cassette. Thus, the FRET fluorophore remains quenched and no fluorescence signal is observed. Specifically, the Invader assay allows for simple multiplexing by changing the probe configurations to achieve the ability to detect large sets of SNPs within target sequences in the genome. Its high specificity and quick reaction time makes it a prime model system for assay development in droplets. Furthermore, to test the capabilities of multiplexing using the pressure controlled valve system, the device must be fabricated from PDMS using soft lithography to allow for deformable channels and valves. Since PDMS is highly porous tends to exhibit air bubble formation at higher temperatures when the heated gas expands, the mild temperature of the Invader assay makes it the ideal candidate to test on the multiplexed valve based PDMS chip system.

2.2 Materials and Methods

2.2.1 Device Design and Fabrication

To satisfy the requirement of achieving multiplexed Invader reactions between eight DNA samples and ten probes, the device was designed specifically with ten probe inlets, two sample inlets coupled with rinsing channels, two pressure relief channels and one carrier oil inlet as shown in **figure 2.2.1**. Devices were fabricated using PDMS SYLGARD 184 (Ellsworth Adhesives) as the base material. Molds were fabricated using standard lithography techniques on 4 inch silicon wafers with SU8-3025 (for fluidic layer) and SPR-227 (for fluidic and valve layers) photoresists (MicroChem Corp). **Figure 2.2.2** shows the fabrication scheme that begins with casting PDMS over the fluidic and valve molds. A thick fluidic layer was first cast using 50g of low PDMS to crosslinker ratio (6:1) and degassed under vacuum conditions for an hour to remove air bubbles present in the PDMS mix. The fluidic layer was then cured for 8 minutes at 80°C and a thin PDMS valve layer was spun on a spin coater (Laurell Technologies, Corp) with high PDMS to crosslinker ratio (15:1) at 1100rpm and cured at 80°C for 4 minutes. The fluidic layer was removed, cut out from the mold and aligned with the valve layer still attached to the silicon mold. Alignment was performed such that all valves covered the channel openings enough to completely close the channels upon pressurization. Adhesion between the fluidic and valve layers was ensured by allowing the assembled chip to bake at 80°C for one hour before the thick fluidic layer attached to the thin valve layer was removed off of the mold. The thin valve layer allowed for valves to be deformed upon pressurization and close off the fluidic layer above with pressures less than 30PSI. Both the fluidic layer and the valve layer were left slightly under baked such that when they were brought into contact and

cured, the crosslinkers from both layers allowed for adhesion. Access holes were then punched using 20 gauge needles (McMaster-Carr) for the inlet and outlet ports and the PDMS device bonded to cover glass (Ted Pella, Inc) after standard oxygen plasma treatment. All devices were kept at 80°C overnight prior to use.

2.2.2 Invader Assay Reagents

All synthetic and genomic DNA targets were packaged and prepared by DuPont Pioneer. Due to the nature of the Invader assay for SNP detection, the synthetic targets were generated to only contain a short sequence containing the SNP as controls to test for the chemistry of the Invader reaction and probes. Thus, all synthetic targets were generated in pairs of sequences (a, b) to differ by only one SNP and thus exhibit complimentary fluorescent signals. All a sequences were synthesized to correspond to Redmond Red alleles, while all b sequences to FAM alleles. Subsequently, each pair of synthetic targets was only tested against the single probe for the particular sequence coded by the synthetic targets.

Bench top Invader assays were performed at 63°C for 30 minutes and optical detection was measured after each cycle of 30 seconds thus generating real-time plots of fluorescence as the Invader reaction took place. All genomic targets were amplified using multiplexed PCR with 10 primers that amplified the 10 regions of interest prior to the Invader assay. This ensured that the final fluorescence read out for Invader reactions was high for genomic samples. Reagents for the multiplexed PCR included FastStart Taq DNA polymerase (Roche), 10x PCR buffer with 20mM MgCl₂ (Roche), 10mM dNTPs (Roche) and corresponding primers (IDT). Amplified genomic DNA targets were denatured at

100°C for 20 minutes prior to the assay, then snap-cooled and kept on ice until use. The Invader assay was carried out using Invader probes specific to the synthetic and amplified genomic targets, Cleavase XI FRET mix (Applied Biosystems), Cleavase XI Enzyme and MgCl₂ solution.

For device operation materials, the carrier oil used to separate aqueous droplets consisted of FC40 (3M) with 1H, 1H, 2H, 2H-perfluoro-1-octanol (PFO) (Aldrich) in a ratio of 5:1 by volume. Oil and probe mixes were assembled off chip, loaded into 0.02 inch Tygon® microbore tubing (Cole-Parmer) and connected with the device through inlet ports using 23 gauge needles (McMaster-Carr). 10µL of DNA each were loaded into PTFE tubing (Cole-Parmer) and connected with the device through sample ports.

2.2.3 Optical Detection and Heating Setup

The optical setup used was previously described by Rane et al. [16]. To achieve isothermal incubation, the microfluidic device was mounted on top of a Peltier heating plate coupled with a PID controller. The Peltier device was mounted in a machined aluminum heat sink connected with a cooling system and the incubation region of the chip lies atop the Peltier plate. The detection region protrudes away from the heating plate and was made accessible to the excitation laser through a detection window while the chip itself is kept at 63°C for optimal Invader activity. This design allows for concurrent incubation and detection and therefore gives the device continuous flow and high throughput capabilities. Laser excitation came from two sources – 488nm and 552nm (Coherent OBIS Lasers) – to excite the FAM and Redmond Red fluorophores present in FRET cassettes of the assay. Dual band detection was achieved with two avalanche photodiodes (APDs) at

506-534nm and 608-648nm. Together, the system was built to perform confocal fluorescence spectroscopy for two color detection. The optical filters used for the FAM and Redmond Red channels were 520/28 and 628/40 (Semrock) respectively. All traces were captured with a neutral density filter of 2 (ND2), allowing for 50% transmission. Data traces were captured using a custom LabVIEW programmed GUI in discretized files of 2 to 10 minutes each, and later concatenated together for analysis in MATLAB. Photon counts were collected in 10 millisecond bins. Optical focusing was done in the middle of the channel highlighted in the detection region (**figure 2.2.3**), and the laser point was focused 10 μ m into the channel from the bottom surface of the channel (in contact with the valve layer).

2.2.4 Microfluidic Device Operation

All valve ports were first connected via Tygon® tubing and needle tips to a pressure control box interfaced with custom MATLAB scripts (MathWorks) to govern the opening and closing of solenoid governed pressure switches. Hence, the time and duration of pressurization and subsequently valve opening was controlled. Valves were operated at pressures ranging between 20 and 30 PSI on average. The carrier oil was loaded through the carrier port (**figure 2.2.1**), and allowed to flow through the entirety of the device before any droplets were generated. Droplet generation was controlled using custom MATLAB scripts with numerical sequences that corresponded to the numbered valves and specified the opening times of each valve, allowing small volumes (nanoliters) of sample and probes to be generated and mixed. Oil was flown in between droplets to act as a separator and to index the droplets.

To ensure that the assay detection was still functional at elevated temperatures, Invader reactions were first performed on bench top and the positive signals confirmed. The samples were then loaded onto the chip and digitized into nanoliter droplets by adjusting the valve opening times. Once sufficient droplets had been generated, the device would be mounted onto the Peltier heating system and incubated at 63°C. Fluorescence read out was performed with the confocal set up at the detection region while droplets were flown past using carrier oil. The general workflow is illustrated in **figure 2.2.3**. Samples and probes were loaded into respective channels, and digitization into smaller, nanoliter sized droplets using pressure controlled, deformable PDMS valves. Similarly, once the droplets reach the probe inlets, reagents mixes containing specific probes were injected into the sample droplet. The multiplexed droplets would then be flown downstream into the incubation channels before emerging at the confocal read out point. Prior to all experiments wherein any aqueous form of sample or reagent was loaded, the fluidic channels within the device were all flown through with carrier oil. This acts to remove any empty air pockets from the channels and provide the droplets with a homogeneous medium to be generated in.

2.3 Results and Discussion

2.3.1 Bench Top Invader Assay Verification

Bench top multiplexed PCR for the genomic DNA and Invader was first performed on bench top using a real-time machine (Bio-Rad CFX96 TOUCH SYSTEM) for all samples against all probes. **Figure 2.3.1** illustrates the results of the Invader assay performed off chip for synthetic targets (**A**) and genomic targets (**C**). Due to the nature of

SNP detection and the samples we received, all genomic and synthetic targets were homozygous for either the FAM labeled allele or the Redmond Red allele. A typical trace observed from the real-time data captured off chip is shown in **(B)** during which the Invader assay was allowed to run for 30 minutes. As evident from the traces in **(B)**, the genomic samples quickly reached a steady fluorescence level after incubation at 63°C for approximately 6 minutes and there was a significant difference between the end point FAM and Redmond Red intensities. Following confirmation of the correct allele call outs determined from the real-time data, the bench top incubated Invader assay products were loaded onto the chip via the sample inlet ports (**figure 2.2.1**) for end point fluorescence measurements. Uniform droplets were generated from bulk samples, observed and confirmed to be intact and homogenously sized under an optical microscope. The droplets were pushed downstream through the incubation region by carrier oil and continuous droplet generation while the device was heated at 63°C. Optical detection was then performed using the confocal set up. Signal to noise analysis was done after data files were concatenated. A manual threshold was defined to separate the signals from the background, and an average photon count of the signal and background was calculated. The recorded signal traces supported the prior observations that uniformly sized, intact droplets with very high signal to noise ratios (around 924:1 for the FAM channel and 175:1 for the Redmond Red channel) could retain their fluorescence after incubation at 63°C. Most importantly, this result indicated that the fluorescent chemistry of the Invader reaction and subsequent signal read out was unhindered at the elevated temperature to the extent where high signal to noise ratios could still be achieved.

2.3.2 On-Chip Invader with Synthetic Targets

Synthetic targets were prepared together with the appropriate Invader reagents and probes in separate tubes on bench top and then loaded onto the chip. Since each probe corresponds to one pair of synthetic targets, the pair of targets were loaded through the two sample inlets and the probe through one reagent inlet. Following the verification of one set of data, another pair of synthetic targets were then loaded. Sample inlets were rinsed in between with water by opening the valve controlling the rinsing channel, thereby allowing water to exit without proceeding further downstream into the chip and preventing contamination between samples. When coupled with an automated loading system, this sample inlet design offers the capability of undergoing automated, high throughput SNP screening. The injected probes and genetic target samples were mixed with the samples via turbulent flow during passage through the serpentine channels downstream [17]. A representative recorded fluorescence trace after incubation is shown in **figure 2.3.2 (A, B)**. Sequences of each pair of synthetic targets were generated in an asymmetrical fashion – a, b, b – where a and b denote the FAM and Redmond Red alleles for each synthetic target pair. This ensured the resulting droplets' identities could be readily determined from the fluorescence data based on which synthetic target was generated as a duplicate. Multiple trains of droplets containing synthetic DNA were collected and its data corrected for APD. Droplets of the same content were grouped using MATLAB. The resultant ratios of FAM signal to Redmond Red signal calculated for each droplet was used as an indication of the SNP allele present in the synthetic target pairs. An average of all the ratios for the same set of droplets was then calculated and a final allele call out was determined based on this ratio. A ratio greater than 1 would result in a FAM allele, while a ratio smaller than 1 would

result in a Redmond Red allele. **Figure 2.3.2 (E)** illustrates the synthetic targets' droplet intensities plotted based on their FAM and Redmond Red intensities where the vertical line represents the separation between the allele call outs. All synthetic targets exhibited ratios of greater than 2 or less than 0.5 – indicating that the positive allele fluorescence was greater than twice its counterpart. A comparison with the bench top data show that the on-chip Invader assay with synthetic targets achieved a 100% correct call out for each allele.

2.3.3 On-Chip, Multiplexed Invader with Genomic DNA Targets

Following the on-chip synthetic target Invader reactions, 8 genomic DNA samples were multiplexed with 10 Invader probes for 10 different SNP sites and the Invader assay was carried out on chip. Multiplexing was performed as described above, and trains of 10 droplets (one DNA sample with the 10 probes) were generated continuously. A representative trace is shown in **figure 2.3.3**, and results from the on-chip multiplexed Invader assay with a final SNP read out for each genomic sample with each probe in conjunction with an averaged ratio of FAM to Redmond Red signals calculated for multiple traces of the same set of sample/probe combinations is shown in **(D)**. A ratio greater than 1 gave rise to a call out of the FAM allele and similarly, a ratio less than 1 was the Redmond Red allele. Of the 80 combinations, only two resulted in a mismatched call when compared to the bench top results. However, these two mismatched call outs both exhibit FAM:Redmond Red ratios that are very close to 1, suggesting that end point measurements for these particular cases may not represent the most effective way for genotyping. Furthermore, the standard deviations calculated for $n = 4$ per DNA/probe combination were

all within the margin of error, suggesting that the signals observed and consequent allelic call outs were robust.

2.4 Conclusions

We have successfully demonstrated a robust microfluidic system capable of performing continuous flow, high throughput multiplexed Invader assay. Generation of droplets in the device remained homogenous throughout experiments and successful mixing and merging of genomic sample droplets with probe reagents was evident through the high post incubation signals observed. Allele call outs were correct for the majority of cases, with the exception of two mismatched instances in a sample pool of 80. It was unlikely that this mismatch resulted from improper mixing or problems with the droplet, as the call out ratio was determined from an average of at least four sets of the same repeated condition with small standard deviations within the margin of error. Thus, it could have been due to the different binding affinities of the Invader probes with different genomic sequence regions. This device offers a novel means to conduct SNP screening for maize, and for future studies can be interfaced with an automatic, serial loading system [18]. As such, this device not only eliminates the need for manual assembly of 80 multiplexed reactions, but also reduces the chance of contamination as well as cost of reagents by reducing the reaction volume from 10 μ L down to 10nL. Furthermore, the outlet port of the device can be easily interfaced with Tygon® tubing such that droplet reactions can be collected and pooled together off chip for future reactions or storage.

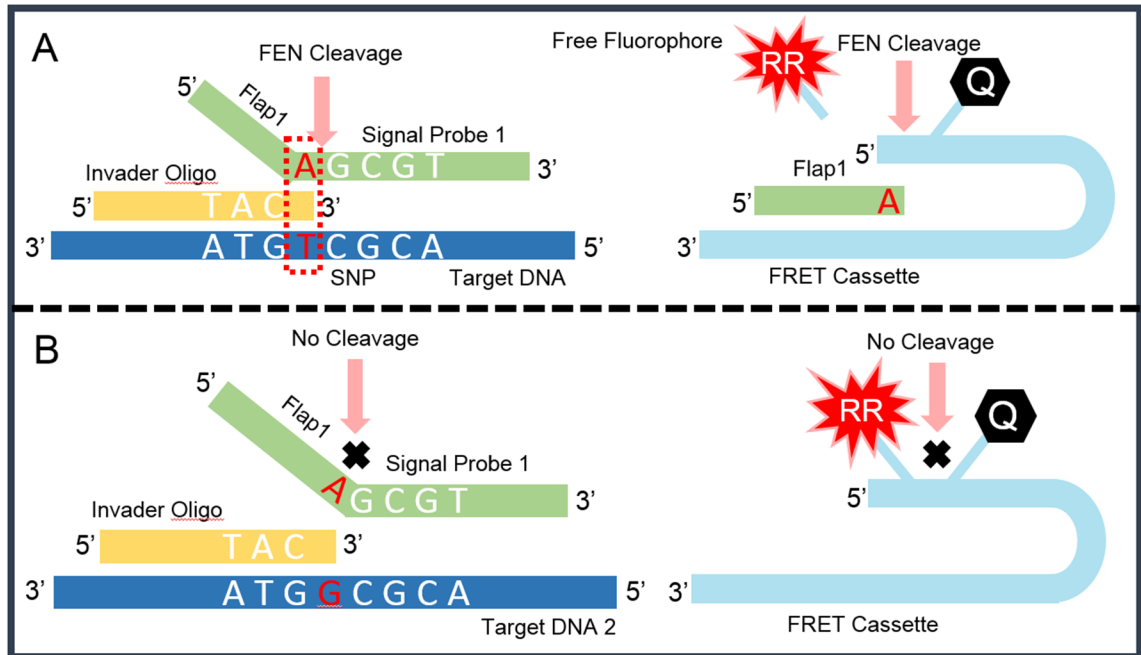


Figure 2.1.1. Basic Invader assay.

- (A) An illustration of the primary reaction that occurs in an Invader assay (right). The Invader oligo (yellow) has a complimentary sequence to the target DNA up to but not including the SNP. Once the Invader oligo is aligned with the target DNA, signal probe 1 (green) with the complimentary sequence containing the SNP combines with the Invader-target DNA complex and forms a tripartite structure. FEN cleavase, upon recognizing this highly specific tripartite structure, cleaves the signal probe 1 at the site marked by the pink arrow thereby liberating flap1. Flap1 forms a similar tripartite structure with the FRET cassette in a secondary reaction. This structure is again recognized by FEN, and cleaved at the site indicated by the pink arrow, releasing the fluorophore from the FRET cassette.
- (B) A single mismatch at the SNP site causes an incorrect tripartite structure formation which is unrecognized by FEN. Flap1 remains attached to signal probe 1 and the subsequent fluorescence remains quenched on the FRET cassette.

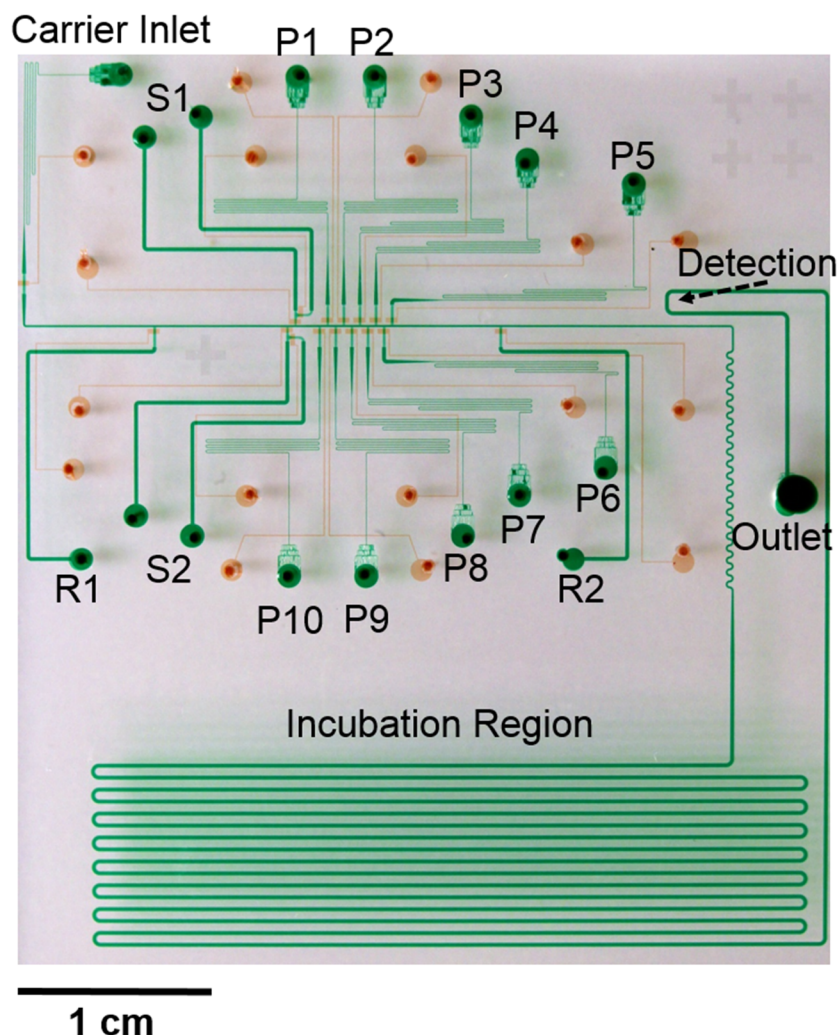


Figure 2.2.1. Microfluidic device for Invader reaction.

Photograph of microfluidic device used to perform Invader reactions on chip. The fluidic layer of the device is shown in green while the valve layer is shown in red. The thin valve layer lies below the fluidic layer, such that when pressure is applied through the red ports the PDMS is forced to deform upward, closing the fluidic channel above. All 10 probe inlets are prefixed with P, pressure relief channels are prefixed with R and sample inlets are prefixed with S. Each sample inlet contains a pair of green ports as shown above. The green port on the left is the sample inlet channel, while the port on the right is the rinsing channel.

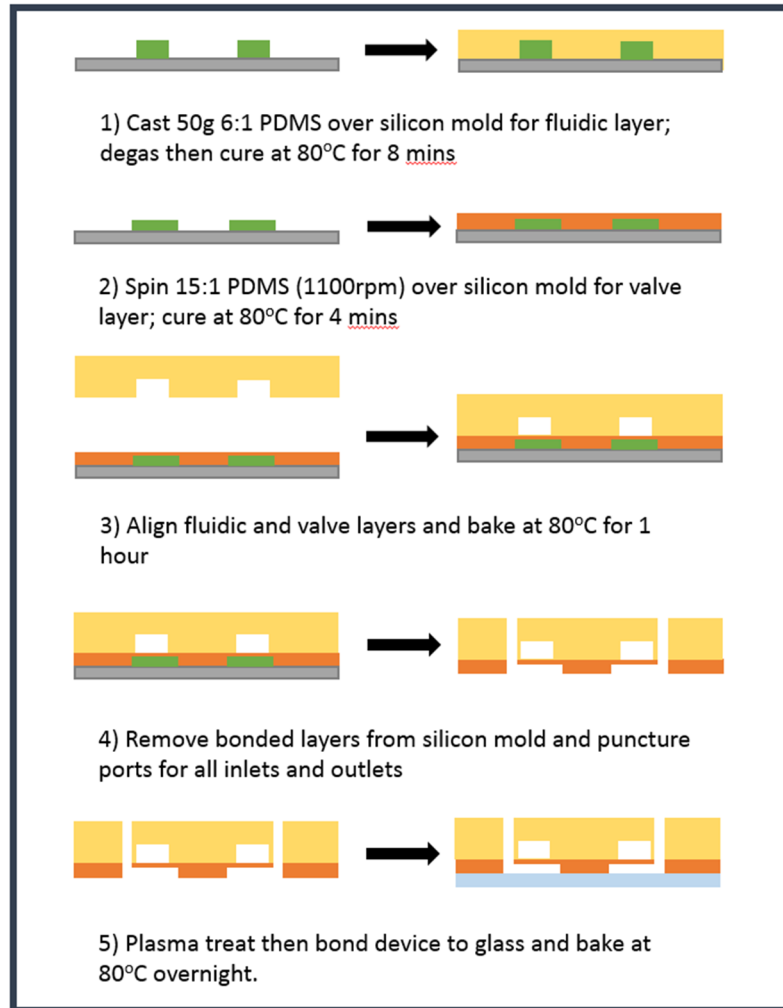


Figure 2.2.2. Fabrication schematic of Invader Device

Illustration of the fabrication scheme involved in the generation of a single device using a silicon mold (grey) generated via standard lithography.

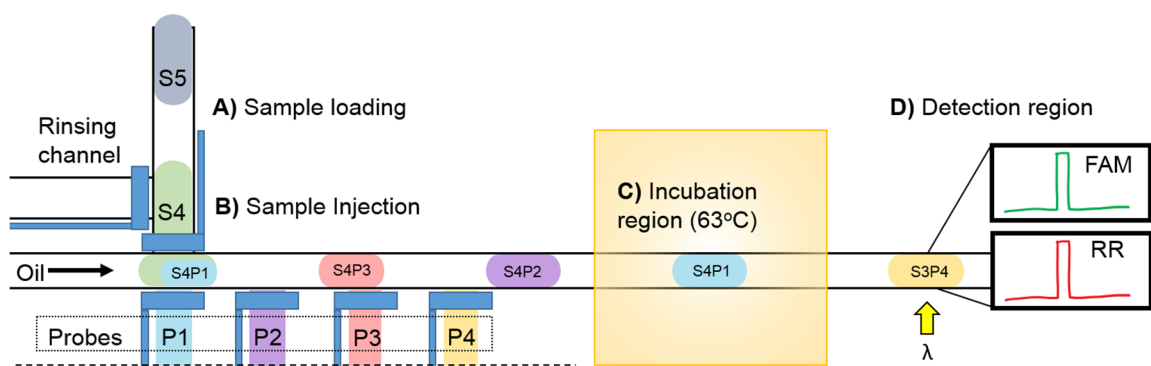


Figure 2.2.3. Simplified and linearized workflow for microfluidic device system

Illustration of the general workflow for the entire reaction system of a droplet in a linear format. Shown in region A is the sample loading, whereby different sample DNAs are sequentially loaded onto into the device. The rinsing channel is used to wash out the sample channel in between to prevent cross-contamination. Point B is the sample injection point where DNA containing droplets are generated and probes are injected into the droplets. Droplets are then flown down into the incubation region held at 63°C (point C) and finally to the detection region (point D) where laser excitation and confocal detection in the FAM and Redmond Red channel occur.

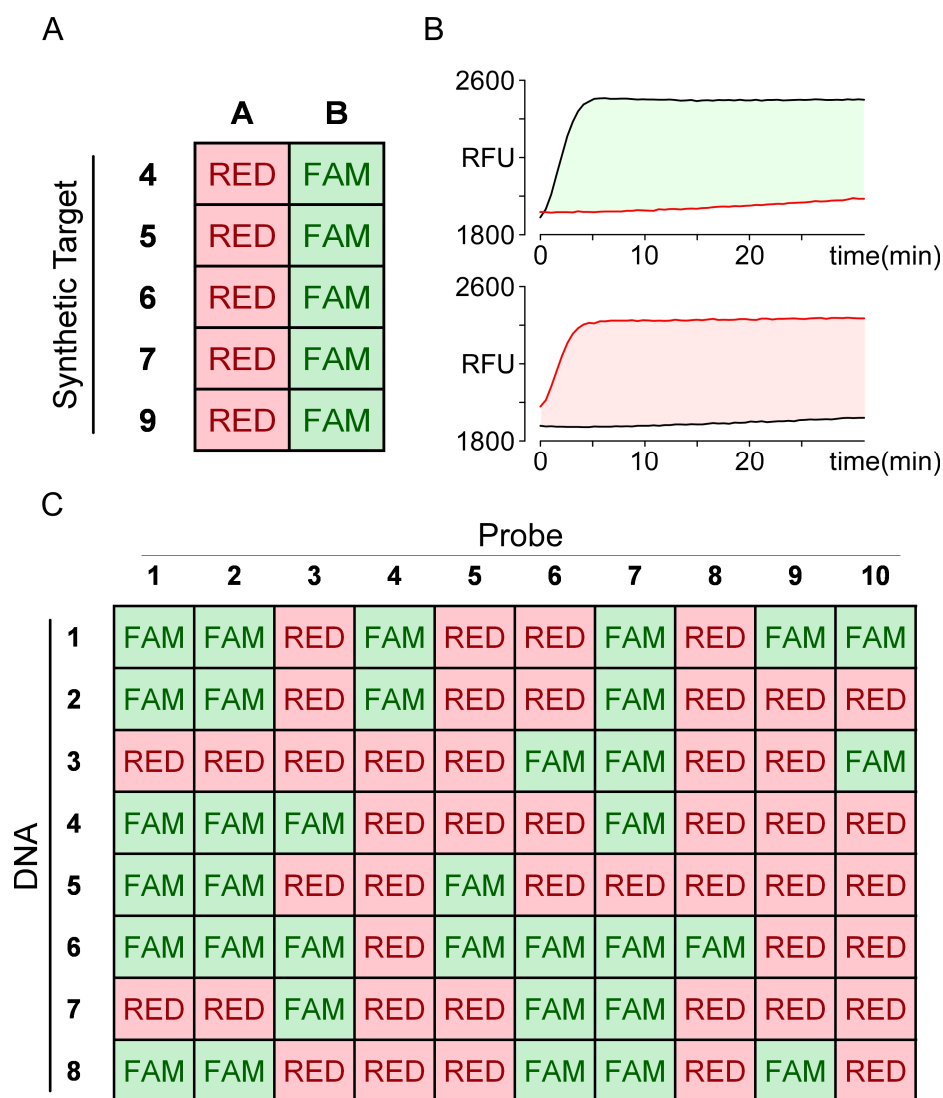


Figure 2.3.1 Bench top Invader assay results

(A) Bench top Invader read out of synthetic target pairs with their corresponding probes.

FAM represents the allele corresponding to the probe containing the FAM fluorophore, and vice versa for RED as Redmond Red. Allele call outs were correct for 100% of the cases.

(B) Representative curves observed from the real-time machine. The reaction was kept at 63°C and a fluorescence reading was taken every 30s.

(C) Bench top Invader read out of multiplexed genomic targets with all 10 probes.

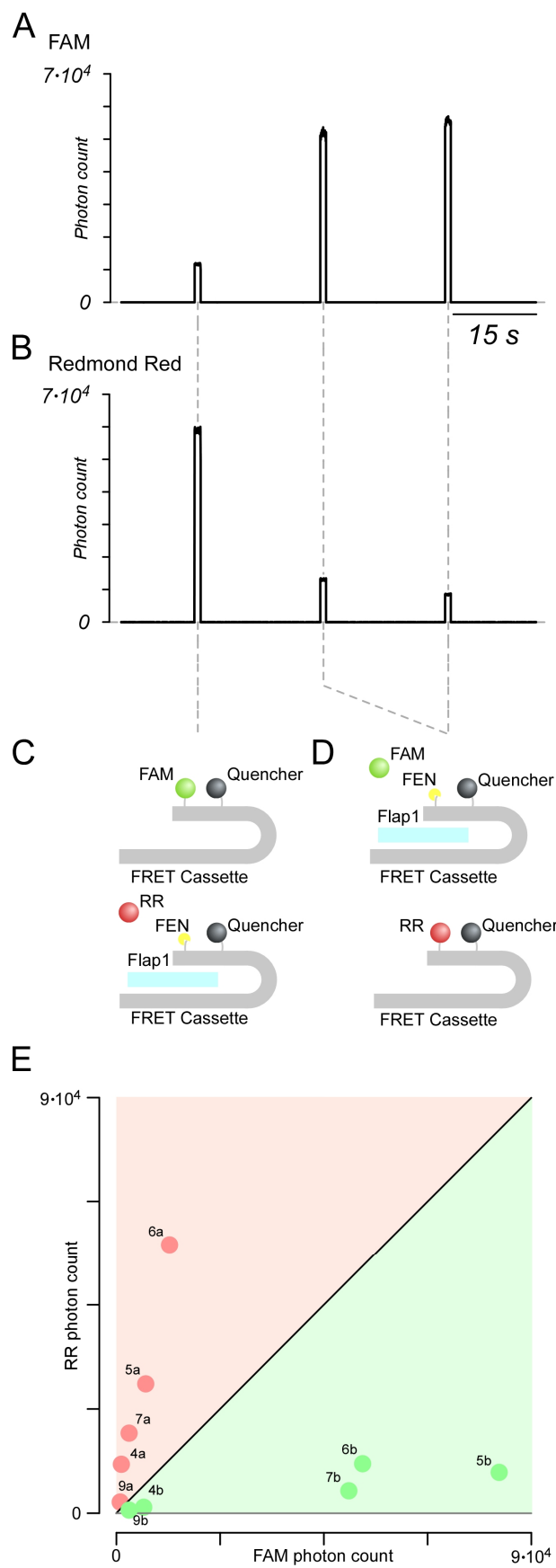
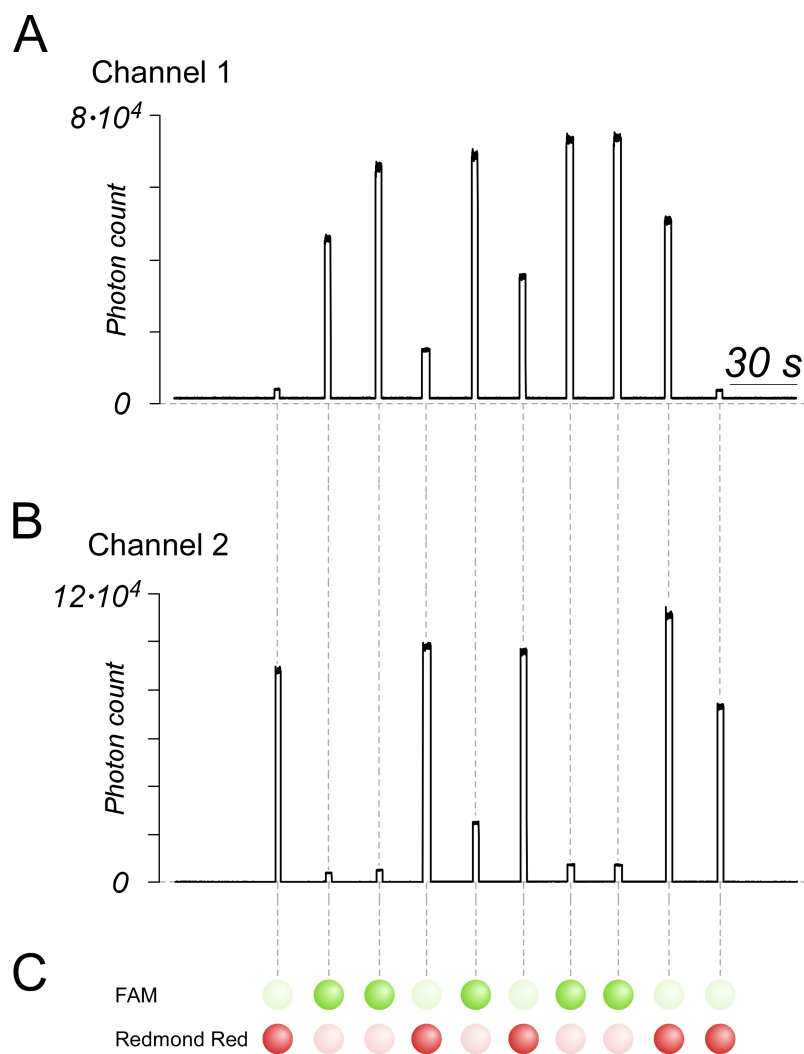


Figure 2.3.2 On-chip Invader assay of synthetic targets

- (A) Representative APD trace of the FAM fluorescence as observed from real-time read out of the Invader assay on chip using synthetic targets. All droplets were generated in the same asymmetrical sequence (a, b, b) such that the latter two peaks observed correspond to droplets containing the same content (synthetic target type b containing the FAM allele).
- (B) Representative APD trace of Redmond Red fluorescence.
- (C) Illustrations of the chemical reaction states in the Invader reaction corresponding to the droplets observed from the positive Redmond Red fluorescence read out. Here, only the flap associated to the Redmond Red SNP is cleaved by FEN, and therefore the secondary reaction only occurs with the Redmond Red FRET cassette. As a result, only the Redmond Red fluorophore is freed from the quencher and fluoresces.
- (D) Illustrations of the chemical reaction states corresponding to the positive FAM droplets.
- (E) Methodology used to determine allele call outs based on end point fluorescence confocal measurements on chip. For each droplet, the ratio of FAM to Redmond Red photon count was calculated for all repeats and call out was determined by whether the ratio was greater or smaller than 1. The black line represents the 1:1 ratio line where FAM photon count is equal to VIC photon count.



D

		Probe									
		1	2	3	4	5	6	7	8	9	10
DNA	1	20.72	17.49	0.091	29.26	-2.721	-0.108	34.59	-0.022	19.49	13.41
	2	16.47	14.66	0.167	14.36	-4.639	-0.008	18.37	0.066	0.096	0.193
	3	1.042	0.122	0.139	0.013	-0.067	12.65	18.27	0.068	0.142	1.043
	4	12.99	10.57	1.785	0.268	0.306	0.399	19.67	0.095	0.088	0.152
	5	7.493	5.524	0.18	0.932	19.86	1.148	0.159	0.025	0.488	0.131
	6	15.45	14.53	4.144	0.002	22.45	17.67	7.189	1.051	0.152	0.441
	7	0.958	0.893	4.574	0.002	0.076	18.86	17.45	0.446	0.104	0.249
	8	13.17	12.42	0.312	0.004	-0.011	18.35	17.34	0.12	2.445	0.318

Figure 2.3.3. On-chip Invader assay of genomic targets

- (A) Representative APD trace of the FAM fluorescence as observed from real-time read out of the multiplexed Invader assay on chip using genomic targets. All combinations were generated by first making the DNA containing droplet, and injecting individual probe mixes into the existing DNA droplet later downstream. Droplet sequences of 10 probes in sequential order were used for all genomic targets.
- (B) Representative APD trace of the Redmond Red fluorescence.
- (C) Illustration of corresponding positive fluorophores based on APD read out
- (D) Call out chart for all 80 multiplexed reactions with 8 genomic DNA samples against 10 Invader probes. Allele type is displayed in the chart as either green (FAM allele) or red (Redmond Red allele). Call out was determined by taking the average ratio of fluorescence intensities for FAM:Redmond Red, and are shown in the chart. Of 80 averaged reactions, only two resulted in a mismatched call (DNA3 with probe 1 and DNA5 with probe 6).

CHAPTER 3:

Allele Discrimination using on-chip PCR

While the Invader assay is a powerful and robust way of allelic discrimination through SNPs, the high specificities and signal to noise ratios for real genomic targets were only achieved with pre-amplification on bench top. The off-chip multiplexed PCR reaction prior to all genomic sample Invader reactions served to amplify only the SNP sequences that were specific to the probes. Thus, although the microfluidic device developed for on-chip Invader allow for much faster and cheaper genomic screening time, it is still limited by the pre-amplification step that requires off chip PCR.

The aim of this chapter is to develop a platform that is capable of detecting multiplexed SNP reactions without the use of bench top pre-amplification. Here, a microfluidic device was developed to perform on-chip PCR tested using a Taqman based allele discrimination assay on genomic DNA extracted from maize. The nature of Taqman PCR forgoes the need for pre-amplification. First, the device was fabricated following the schematic shown in **figure 2.2.2**, interfaced and operated with the pressurizing system and mounted onto a two temperature zone based heating system for thermocycling. Similar to the approach taken by the Invader studies, this chapter begins by performing PCR on bench top for three DNA targets against four Taqman probes. Optical detection of the bench top amplified assay products was then tested on various iterations of the microfluidic device. Finally, on-chip Taqman PCR was performed by loading bench top assembled reaction mixes with positive genomic samples, such that the thermocycled droplets could be

collected via Tygon® tubing and pooled together off chip. The collected sample was then analyzed using gel electrophoresis to determine whether the correct amplification had occurred.

3.1 Taqman Based PCR Assay for Allele Discrimination

The reaction selected to conduct on-chip PCR involves the use of Taqman probes and the reaction shown in **figure 3.1.1**. Similar to the Invader assay as described above, Taqman assays function to detect SNPs by relying on a combination of PCR amplification and polymerization of Taqman probes with the sample template. Due to the nature of the assay being a polymerase dependent amplification assay, it requires a ‘hot start’ step during which the polymerase is activated to prevent non-specific amplification. The reaction mix containing the Taqman probes, polymerases, primers and templates are then thermal cycled at optimized temperatures to allow for denaturation, amplification and annealing. For our specific assay, each probe appears as a set containing the same specific sequence that differ at only one base pair (the SNP), quenchers and two fluorophores that correspond to the two SNPs. The polymerization and fluorescence occur concurrently as shown in **figure 3.1.1 (C)**. If the DNA template contains the SNP of the VIC probe, the probe would hybridize with the DNA template and as the single stranded template begins to polymerize, the fluorophore would be cleaved by polymerase and thus freed from the quencher and a VIC signal would be seen. Taqman PCR, like the Invader assay, allows for simple multiplexing by design of probe configurations and can be used to detect large sets of SNPs within target sequences. It also has the advantage over the Invader assay in that its PCR amplification step negates the need for off chip amplification, and thus contains all aspects of genotyping

on chip. This makes it a powerful tool for tasks that require high-throughput screening as it reduces the time and reagent costs needed for pre-amplification off chip. However, as described in Chapter 1, PDMS behavior is highly susceptible to changes at higher temperatures. The thermocycling step required for PCR thus poses as a challenge in developing PDMS-based PCR microfluidics platform. This chapter will serve to describe our approaches from the design and testing perspectives to achieve thermocycled Taqman based PCR SNP detection in a PDMS-based microfluidics platform.

3.2 Materials and Methods

3.2.1 Device Design and Fabrication

Device fabrication was done following the schematic presented in **figure 2.2.2**. Silicon molds were created separately for the valve and the fluidic layers. 4 inch silicon wafers were spun with SU8-3025 and SPR-227 (Microchem Corp) at varying spin rates to achieve thicknesses corresponding to the valve and fluidic layers. The resist comprising the valve layer was made to be $\sim 35\mu\text{m}$ as examined with a profilometer, and the fluidic layer was fabricated to be $25\mu\text{m}$ in the areas where valves close, $50\mu\text{m}$ at the inlets, $100\mu\text{m}$ at the generation channel region and stepped up to $200\mu\text{m}$ for the rest of the incubation and outlet (**figure 3.2.1**). The increased incubation channel height served to minimize droplet contact with PDMS side walls, thereby reducing the surface area over which evaporation will occur. All device iterations were made from the silicon molds using PDMS from SYLGARD 184 (Ellsworth Adhesives) with 15:1 crosslinker to PDMS ratio spun on at 1100rpm as the valve layer and 10:1 crosslinker to PDMS (50g, degassed) as the fluidic layer. Assembly of the two devices follows the same procedure as that mentioned in chapter

2. After access hole had been punched, the assembled device was mounted onto glass (Ted Pella, Inc) with standard oxygen plasma treatment. A photograph of the most recent iteration of the device design is shown in **figure 3.2.1**. Tapered, triangular inlets as seen in **(i)** were used instead of regular rectangular ones to reduce the dead volume, which is defined as the volume present after droplet formation in the generation channel but remains stuck in the nozzle.

Due to severe evaporation occurring as a result from the lengthy incubation channels, modifications to this design were added in attempt to reduce water vapor loss and thus subsequent changes in salt concentrations in the droplets containing PCR reaction mixes. An additional glass layer was mounted on top of the PDMS slab, covering the incubation regions to prevent water loss through the top of the device. This was done so using the same oxygen plasma treatment conditions as described previously. Furthermore, the sides of the device at the incubation regions that were still exposed to air due to the thickness of the PDMS slab were covered with epoxy glue (3M) and cured at 80°C overnight prior to use. The purpose of this was twofold: first, the epoxy acted to isolate the PDMS incubation region from contact with air to prevent escape of vapor, thereby saturating the device over time and reduce evaporation effects of subsequent droplets. Second, by isolating the incubation region from air and placing the device under vacuum to degas prior to use, we minimized the amount of gas present in the highly porous PDMS device and reduce prevent expansion of air upon heating which leads to subsequent air bubble formation in the channels. All inlet and outlet ports were sealed from contact with air using Magic tape (Scotch), such that when the chip was removed from vacuum conditions, air could not re-enter the device.

3.2.2 Taqman PCR Reagents

Three genomic targets were packaged and prepared by DuPont Pioneer. PCR reagents included four sets of Taqman probes specific to four SNP sites, and KlearKall Master mix (LGC) containing KlearTaq hot start DNA polymerase, salt buffers, dNTPs and ROX dye as reference.

The carrier oil used for on-chip experiments consisted of FC40 (3M) with 1H, 1H, 2H, 2H-perfluoro-1-octanol (PFO) (Aldrich) in a ratio of 5:1 by volume. Surfactant oil used for on-chip experiments were made from the FC40:PFO (5:1) mix as described previously with 2% RAN surfactant (Sigma-Aldrich) by weight. The addition of surfactant ensured droplet stability and prevented unwarranted fusion between droplets which is critical at temperatures as high as 95°C. Oil and surfactant mixes were assembled off chip, loaded into Tygon® microbore tubing (Cole-Parmer) and interfaced with the device through separate oil and surfactant ports. All reaction mixes were loaded into PTFE tubing (Cole-Parmer) and connected with the device via inlets punched in the PDMS device. Final gel electrophoresis was performed in 2% agarose gel (by weight in 1x TAE buffer) in 1x TAE buffer at 120mV and 90mA for 50 minutes.

3.2.3 Optical Detection and Two-zoned Heating

The optical detection set up used was the same as described in chapter 2 [16]. Laser excitation came from the same two sources – 488nm and 552nm (Coherent OBIS Lasers) – corresponding with the FAM and VIC fluorophores present on the Taqman probes. Dual band detection was achieved with two the same APDs to perform confocal fluorescence spectroscopy for two color detection. The filter used for the VIC channel was 575/25

(Semrock) to minimize the effect of the ROX dye present in the buffer and maximize the VIC fluorescence capture. For the thermocycling set up, a passive heat sink was machined to allow for three separate Peltier heaters to be mounted (**figure 3.2.2**). Each of the three heaters was controlled by its own PID system, such that they could operate independently at three separate temperatures. For the purpose of the Taqman assay, only two temperatures were used and the device was designed such that the time scale for incubation at each temperature correlated with the length scale. The Taqman PCR consisted of thermocycling between 60°C for 1 minute, and 95°C for 30 seconds as well as a 10 minute hot start at 95°C. Consequently, the incubation lengths were designed in a 2:1 ratio, whereby 2/3 of the width was mounted atop the 60°C heater and 1/3 was on the 95°C heater. Similarly, a series of long channels that run perpendicular to the incubation channels were designed as shown in **figure 3.2.1** at the bottom left. These channels corresponded to the 10 minute hot start and lie atop 95°C heater (**figure 3.2.2**). In this manner, the time spent at each temperature zone can be fine-tuned by adjusting the pressure driving the carrier oil.

Optical detection was performed in a similar manner as mentioned in chapter 2. Droplets exiting the thermocycled incubation regions emerged and flowed past the detection region as shown in **figure 3.2.1 (ii)**. Due to the increased channel height (200µm), laser focusing was done by aiming 20µm into the channel from the bottom.

3.2.4 Microfluidic Device Operation

The same MATLAB script used in chapter two was used to control the pressure governed valve functions of this device. Valves were operated at pressures ranging between 25 and 35 PSI on average, and both oil and surfactant were flown in between droplets to

act as a separator and stabilizer for the droplets. Opening sequence times for the oil and surfactant were optimized to be a 5:1 ratio for optimal performance. Both samples and reagents were loaded in a similar manner to that described in chapter 2, only that for the case of on-chip Taqman PCR amplification, PFTE tubing was also interfaced with the outlet hole as a means to collect the amplified droplets into a 1.5mL Eppendorf tube before running it off chip on a gel. For optical detection studies, asymmetric droplet trains containing bench top amplified positive and null template controls were loaded, along with a sample consisting only of the KlearKall buffer. This was to account for any contributions that the ROX baseline dye made towards the intensity of the VIC fluorescence. The total time of the bench top Taqman PCR was optimized and programmed to be 70 minutes, including hot start and 40 cycles. Hence, pressure variations were adjusted to examine how long a droplet takes from generation to travel to the detection region.

3.3 Results and Discussion

3.3.1 Bench Top Taqman PCR Assay Verification and Optical Detection

Figure 3.3.1 illustrates the allelic discrimination data based on Taqman PCR using two DNA samples – one heterozygous and one homozygous – (SX19 and B73 respectively) against four Taqman probes. As evident from the end point read out taken at the 39th cycle, SX19 remained heterozygous for all probes whilst the other two genomic samples were homozygous. A representative trace is shown in panels (A-C).

Following verification, bench top amplified products were loaded onto the device and discretized into nanoliter sized droplets with the use of valves and flown in through the incubation channels. To check that the fluorescence signal of the bench top amplified

products would not be degraded upon heating, two sets of target genomic DNA were amplified with one probe and four samples (NTC + probe, SX19 + probe, B73 + probe and KlearKall buffer mix) loaded onto chip for confocal fluorescence detection. The KlearKall buffer was added to ensure that the ROX dye present did not drastically alter VIC fluorophore readings. **Figure 3.3.2** shows the results of optical detection of these bench top amplified products after having been flown through the entire device and heated with the two-temperature Peltier system. Average photon counts for all samples were calculated by defining thresholds to separate droplets from noise, then grouping the droplets of the same composition and taking the average of the FAM and VIC photon counts for each set of droplets. Signal to noise ratios were all very high, with the lowest being the negative control to noise ratio for the VIC channel as 15.2. Positive allele signals were compared to their respective negative controls, and were calculated to be 7.51 and 5.08 for the two positive FAM signals respectively, and 4.46 and 2.08 for the positive VIC and homozygous FAM signals (**figure 3.3.2**). This suggests that a positive signal would exhibit a signal to negative control ratio of greater than 2.08 as an end point measurement.

3.3.2 On-Chip Taqman PCR with Genomic DNA Targets

To verify that on-chip thermocycling resulted in the correct amplification products, the on-chip amplified sample droplets were collected and gel electrophoresis was performed to compare with bench top amplified products. The reaction mix containing genomic DNA that was heterozygous for the SNPs was loaded into PTFE tubing and injected into the chip. After enough droplets had been generated such that they approached the outlet, optical measurements of FAM and VIC fluorescence were measured as an

indication of pre-amplification photon counts (**figure 3.3.3 A**). Post-amplification photon counts were collected after 2 hours of thermocycling to exclude droplets which had experienced incomplete cycling. The FAM to VIC ratio of on-chip amplified droplets was calculated to be 0.969, which is very close to 1 and expected of a heterozygous genomic sample. However, without comparison to on-chip amplified negative controls it is difficult to gauge solely from end point measurements whether or not a sample is positive. Gel electrophoresis results are shown in **figure 3.3.3 B**, and suggest that the on-chip thermocycling achieved correct amplification of the desired product, as seen in comparison with the bench top positive control lane. The smearing was original thought to arise due to a high DNA loading concentration. However, dilution of the DNA on another gel resulted in the same smeared bands, suggesting that it is a result of nonspecific amplification byproducts. The position of the smear indicate low molecular weight smears, potentially due to poor temporal and thermal control of the cycling process. Subsequently, the droplets may have spent too long in the annealing or extension steps in the thermocycling process, or the annealing temperature may have been too low.

3.4 Conclusions

This chapter demonstrated promising results for a continuous flow, high throughput microfluidics platform for PCR based genetic read out. With the use of epoxy, higher channel heights and vacuum degas prior to operation, evaporation and air bubble formation within the channels were mitigated, and optical detection of bench top amplified products retained large signal to negative control ratios, suggesting that on-chip endpoint allele read out would be a good way for genotyping. Due to the constraint of having to collect on-chip

amplified product to run gel electrophoresis to verify correct amplification, the current state of the device is limited to amplifying one type of product at a time (i.e. the positive sample). The on-chip amplified sample did, however, present a bright and smeared band mimicking that of the bench top amplified positive control. This implies that the correct amplification is taking place, and that was reflected from the increased fluorescence signals seen from confocal detection. In conclusion, this device provides good groundwork for multiplexed PCR to be built on, and evidence has shown that the correct amplification is occurring, only that nonspecific amplification is also occurring. Thus, future directions for this project include more rigorous characterization of incubation timing. Specifically, the time that droplets spend in each temperature region must be carefully controlled to prevent nonspecific amplification. Furthermore, heating control must also be finely tuned to ensure that the channels are experiencing correct temperatures throughout the length of the device. By adapting on-chip PCR, this device eliminates the need for any off-chip steps. Sample loading can again be coupled with an automated system, and together this platform would then be capable of fully automated, continuous flow and high throughput genetic screening, making it ideal for uses that require cheap and rapid genotyping.

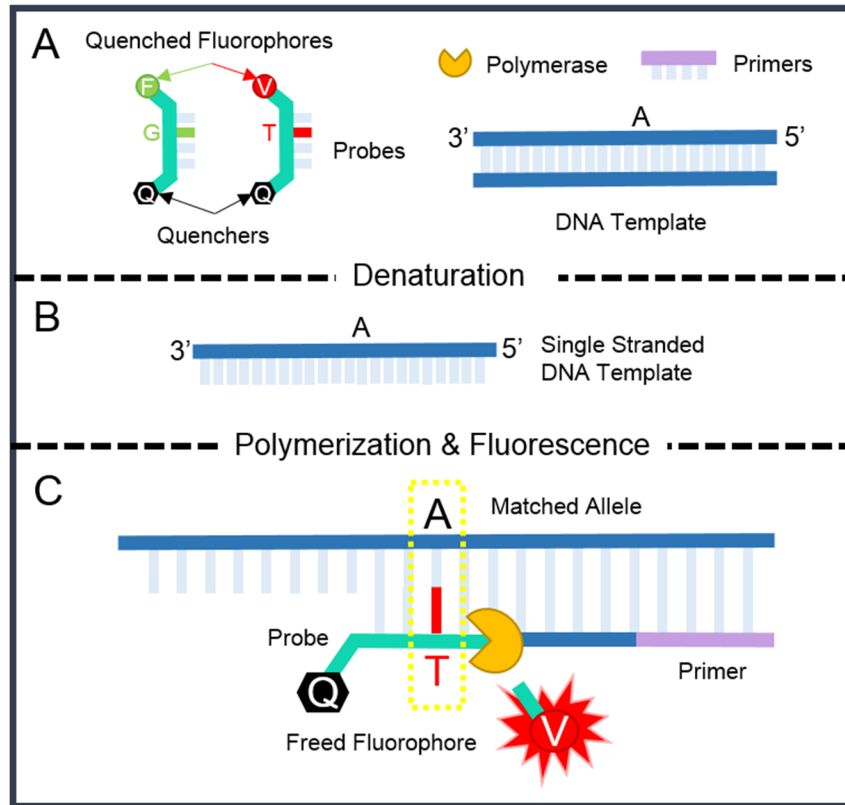


Figure 3.1.1 Overview of Taqman based PCR for SNP detection

- (A) Taqman PCR reagent mix used for all reactions
- (B) Denaturation step used to separate double stranded genomic DNA and expose the sequence for amplification as well as detection
- (C) Polymerization step during which primers have annealed to single stranded template and amplification occurs with polymerase. The Taqman probes will hybridize with the complementary segment of the single stranded template containing the correct SNP such that when the complex encounters the polymerase, the active fluorophore is cleaved off the Taqman probe and free to fluoresce.

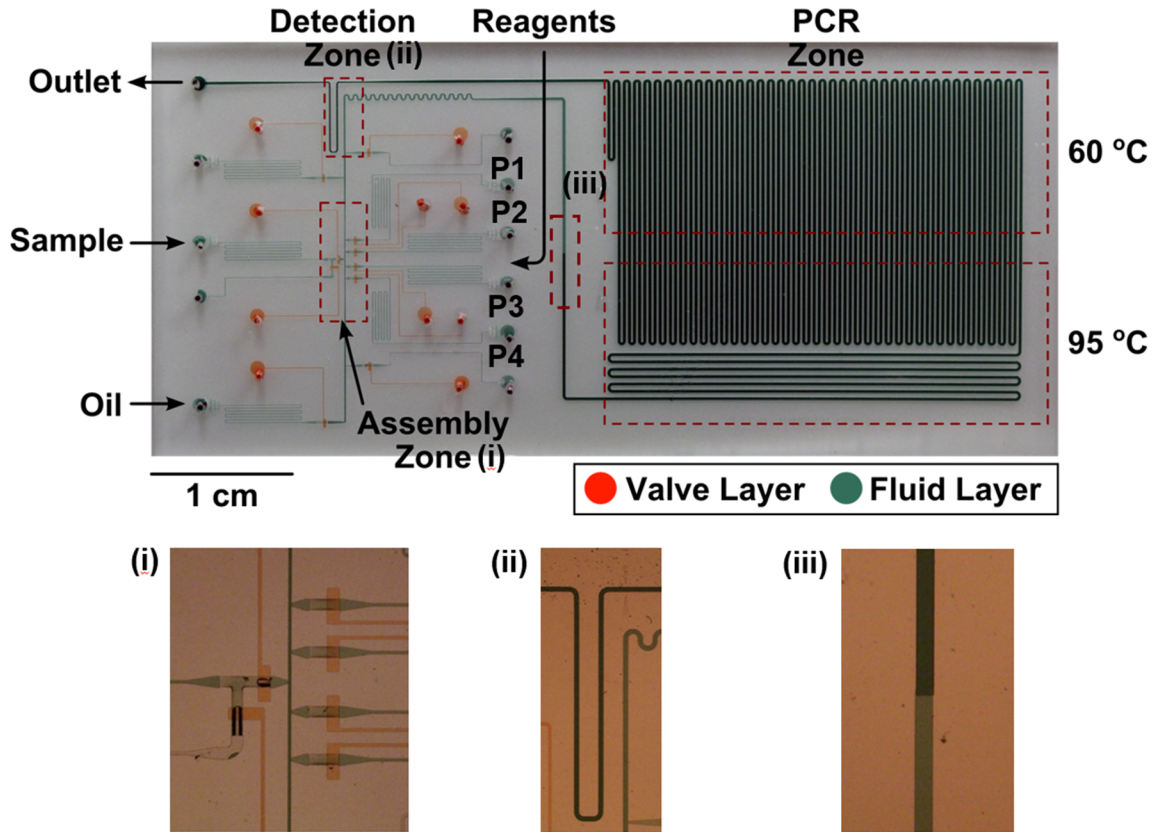


Figure 3.2.1 Microfluidic device for Taqman PCR.

Photograph of microfluidic device designed for Taqman PCR. The fluidic layer of the device is shown in green while the valve layer is shown in red. All 4 probe mix inlets can be seen around the reagent arrow, labeled P and numeric suffixes. Three regions of interest are highlighted: **(i)** is a microscopy image taken of the assembly zone, where the coupled sample-rising channel can be seen towards the left and the four reagent inlets can be seen towards the right, **(ii)** is the detection zone, where the darker green reflects the greater incubation channel height (200 μ m) versus the generation channel height (100 μ m) and **(iii)** is the step up region interfacing the generation region (100 μ m) with the incubation region (200 μ m).

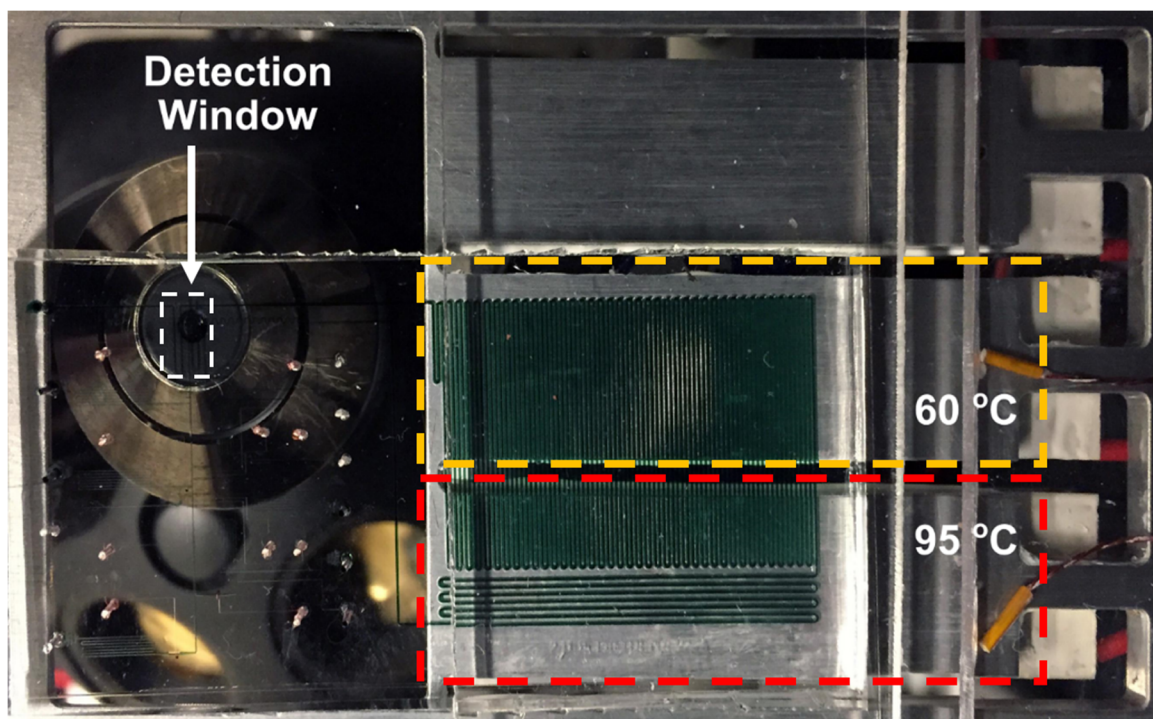
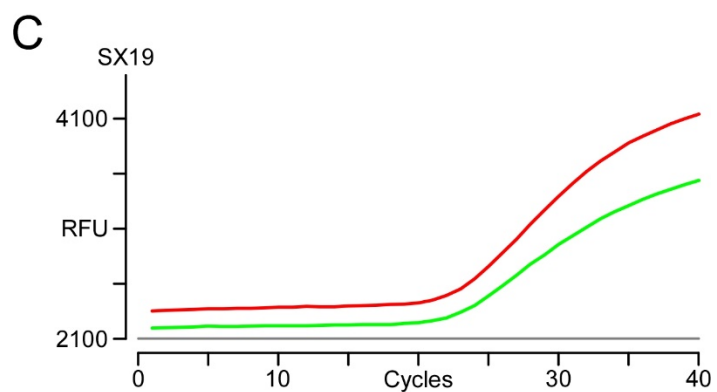
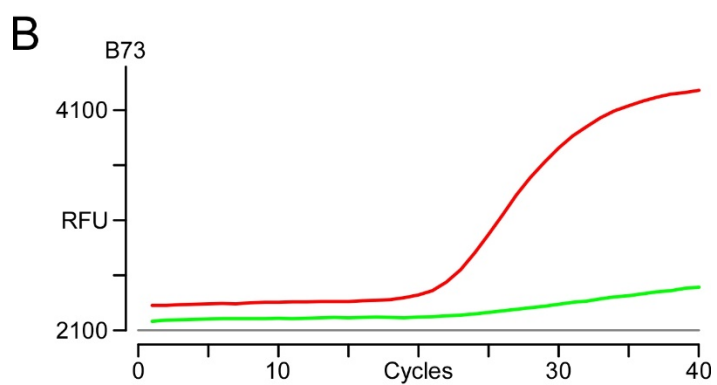
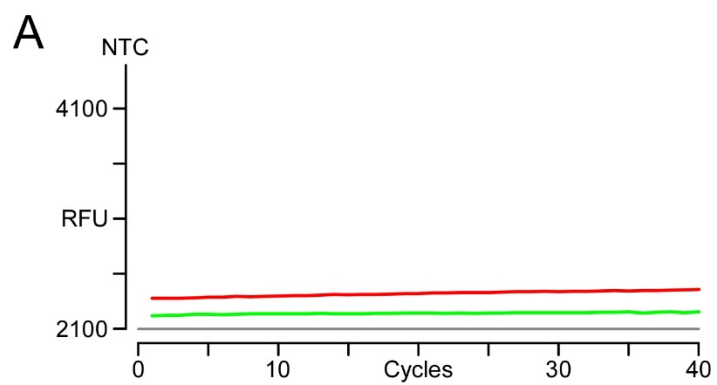


Figure 3.2.2. Photograph of microfluidic device mounted onto heating set up

Photograph showing the overview of the combined chip, heating and optical detection set up. The device is mounted such that the hot start and 95°C denaturation portions of the incubation channel are in contact with the 95°C Peltier heater, and same with the 60°C regions. The aluminum heat sink is designed to allow the top region of the chip containing the detection window to directly come into contact with the objective, thereby allowing for precise focusing of the confocal system.



D

		SX19	B73
Probe	1	F+V	FAM
	2	F+V	VIC
	3	F+V	VIC
	4	F+V	FAM

Figure 3.3.1 Bench top allelic discrimination data for Taqman PCR

- (A) Representative bench top real time traces for no template control (negative control) using probe 2. No amplification is seen from the lack of fluorescence increase in both FAM and VIC channels.
- (B) Representative bench top real time traces for homozygous B73 sample where only the positive VIC signal is seen.
- (C) Representative bench top real time traces for heterozygous SX19 sample.
- (D) Read out chart for all samples multiplexed with all probes. Final allele call was determined from both end point measurements and amplification curves.

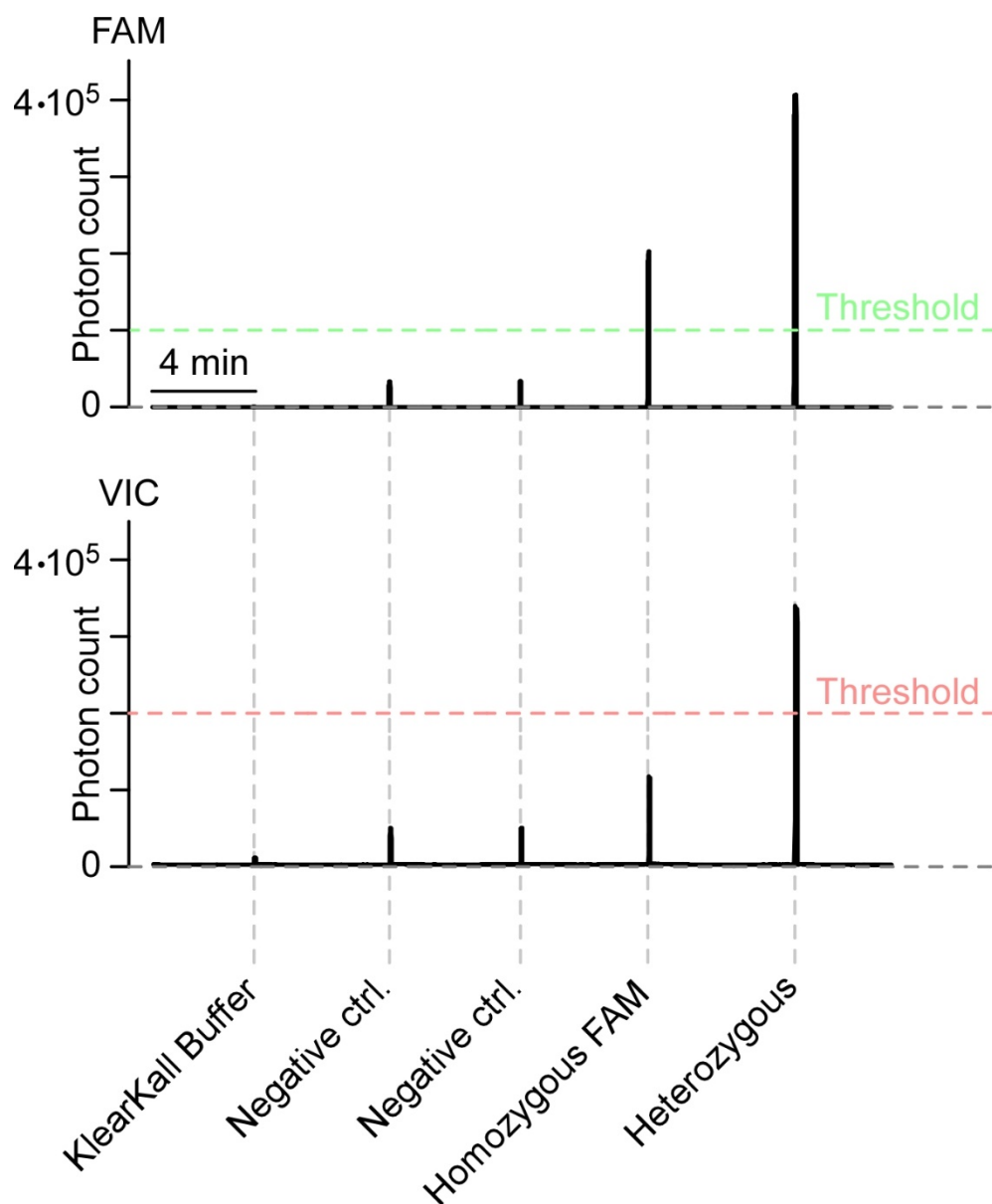


Figure 3.3.2 On-chip optical detection of bench top amplified products

Representative traces showing droplets of specified trains (KlearKall buffer, two droplets of bench top amplified negative control, B73 homozygous sample and SX19 heterozygous sample).

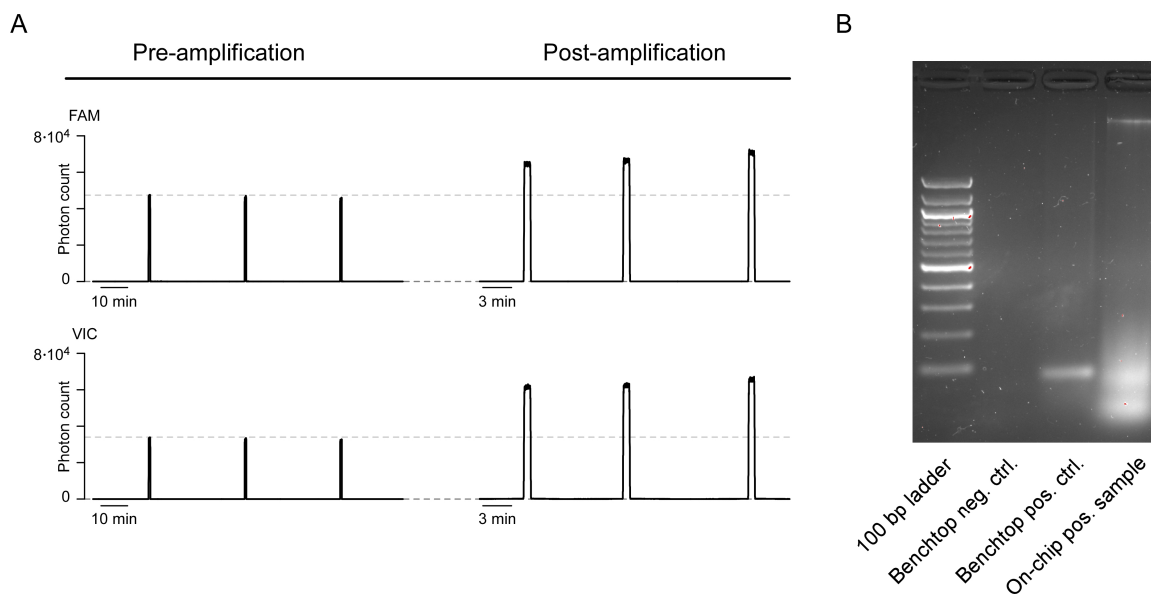


Figure 3.3.3 On-chip amplification and characterization

(A) Representative optical traces recorded from digitized PCR mix with SX19 droplets.

Droplet photon count was measured prior to thermocycling and after thermocycling to determine changes in fluorescence.

(B) Gel electrophoresis photograph of on-chip amplified target (4th lane) compared with bench top amplified target (3rd lane) and its respective negative control (2nd lane).

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Curriculum vitae

Lingshu Liu
110 W. 39th Street, Apt 700, Baltimore, 21210, MD
lliu48@jhu.edu

EDUCATION

- 2014 – 2015 *Master's of Science and Engineering* in Biomedical Engineering
Johns Hopkins University, Baltimore, MD
GPA: 4.0
- 2010 – 2014 *Bachelor of Science* in Biomedical Engineering
Johns Hopkins University, Baltimore, MD
Bachelor of Science in Biomedical Engineering (2014)
GPA: 3.94

RESEARCH EXPERIENCE

- 2014 – present *Master's Thesis Research* in Biomedical Engineering
Johns Hopkins University, Baltimore, MD
Advisor: Tza-Huei (Jeff) Wang, Ph.D.
- Design microfluidic systems for high throughput genetic analysis
- 2011 – 2014 *Undergraduate Research* in Biomedical Engineering
Johns Hopkins University, Baltimore, MD
Advisor: Kevin. J. Yarema, Ph.D.
- Investigate sugar analog toxicity and selectivity for pancreatic cancer cells

TEACHING EXPERIENCE

- 2013 – 2014 *Learning Den Tutor*
Johns Hopkins University, Baltimore, MD
- Tutoring for thermodynamics and statistical mechanics

2014

Teaching Assistant

Johns Hopkins University, Baltimore, MD

Professor: Elizabeth Logsdon, Ph.D.

- Teaching assistant for biomedical engineering practice and innovation lab

PUBLICATIONS

1. Saeui, C. T., Urias, E., **Liu, L.**, Mathew, M. P., & Yarema, K. J. (2015). Metabolic glycoengineering bacteria for therapeutic, recombinant protein, and metabolite production applications. *Glycoconjugate journal*, 1-17.
2. Mathew, M. P., Tan, E., Saeui, C. T., Bovonratwet, P., **Liu, L.**, Bhattacharya, R., & Yarema, K. J. (2015). Metabolic glycoengineering sensitizes drug-resistant pancreatic cancer cells to tyrosine kinase inhibitors erlotinib and gefitinib. *Bioorganic & medicinal chemistry letters*, 25(6), 1223-1227.

HONORS AND AWARDS

2014

Richard J. Johns Award for Outstanding Academic Achievement